Susceptibility to Diet-Induced Atherosclerosis in Transgenic Mice Expressing a Dysfunctional Human Apolipoprotein E(Arg 112, Cys 142)

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Abstract  Transgenic mice expressing apolipoprotein (apo) E(Cys 142), a human defective variant of apo E, have elevated levels of plasma cholesterol, triglycerides, and very-low-density lipoproteins (VLDL). β-VLDL, the biochemical hallmark of the human genetic disease type III hyperlipoproteinemia (HLP), is also present in these mice. This study was designed to determine whether these type III HLP mice have an increased susceptibility to spontaneous or diet-induced atherosclerosis. Three 4-month-old male transgenic mice and three male nontransgenic littermates were assessed for the presence of atherosclerotic lesions in the aorta and mesenteric arteries. No lipid-stained microscopic lesions were visible in the aortas of nontransgenic mice, whereas minimal lesions were observed on the aortic valve stumps of transgenic mice. To magnify the effect of the mutant apo E on the susceptibility of the transgenic animals to atherosclerosis, 8 transgenic and 8 nontransgenic mice were fed a synthetic diet containing 1% cholesterol, 16% fat, and 0.5% cholic acid for 3 months. The diet induced an increase in plasma cholesterol level in both transgenic and nontransgenic mice. However, the increase in plasma cholesterol level in the transgenic mice was all in the VLDL fraction, whereas in nontransgenic mice it was due to increases in both VLDL and high-density lipoprotein (HDL) fractions. Plasma triglyceride levels fell in both groups of mice. After 3 months on the diet, there were compositional changes in the VLDL of both groups, characterized mainly by higher cholesteryl ester content, that resulted in β-migration on agarose gel electrophoresis. Despite similar VLDL lipid compositions, the extent of atherosclerosis differed markedly in the two groups. Nontransgenic mice had small lesions localized only to the aortic valve stumps, whereas transgenic mice had complicated lesions with high cellularity and fibrous caps on both the valve stumps and the aortic wall. These results indicate that the abnormal lipoprotein that accumulates in the plasma of apo E(Cys142) mice has a strong atherogenic potential, and support the hypothesis that β-VLDL is responsible for the development of atherosclerosis in type III HLP.

Key Words  • β-very-low-density lipoprotein • cholesterol • type III hyperlipoproteinemia • transgenic models • hyperlipidemia

Type III hyperlipoproteinemia (HLP) is a genetic disorder of lipoprotein metabolism whose molecular cause is one of several mutations in the gene for apolipoprotein (apo) E that result in the production of a dysfunctional protein or in its absence from the plasma (for review, see References 1 through 3). The function of apo E, a plasma protein associated with several lipoprotein classes, is to bind to the lipoprotein receptors and mediate the clearance of chylomicron remnants, very-low-density lipoprotein (VLDL) remnants, and intermediate-density lipoproteins (IDL). The phenotype of type III HLP is characterized by elevation of levels of plasma cholesterol and triglycerides caused by the accumulation of a specific lipoprotein, β-VLDL, and by the formation of lipid deposits in the skin and the artery wall. Affected individuals are predisposed to premature coronary and peripheral vascular disease. The β-VLDL are composed of both the intestinal chylomicron remnants and the hepatic VLDL remnants. Several reports have indicated that susceptibility to atherosclerosis in people with type III HLP is due to the β-VLDL but there is no direct proof that this lipoprotein can induce atherosclerosis.

Apo E is a polymorphic protein. In humans, three different alleles (e2, e3, e4) at a single gene locus are responsible for six phenotypes, three homozygous (E2/2, E3/3, E4/4) and three heterozygous (E3/2, E4/2, E4/3). A common isoform, apo E2, is defective in binding to the low-density lipoprotein (LDL) receptor, and homozygous carriers may develop type III HLP. Although apo E2 homozygosity is the most common cause of type III HLP, other rare mutations in apo E have been identified that cause a similar hyperlipidemic phenotype. One of these mutations, apo E(Cys142), discovered in a family of Salvadoran origin, is of particular importance for two reasons. First, there is the substitution for arginine by cysteine at position 142 in the middle of the receptor-binding region (amino acids 140 through 150), and second, the hyperlipidemia is present in heterozygous carriers and develops early in life. For these reasons we predicted that the apo E(Cys142) mutation would re-create the hyperlipidemic phenotype in transgenic mice, producing an experimental model of type III HLP. We recently succeeded in producing transgenic mice expressing high levels of apo E(Cys142) from the liver. These mice have a spontaneous hyperlipidemia characterized by an increase in levels of cholesterol and triglycerides and by a dramatic...
change in the lipoprotein profile, from one of mostly high-density lipoproteins (HDL), typical of normal mice, to one based on VLDL, whose accumulation is induced by the retarded clearance of remnant lipoproteins carrying the dysfunctional apo E. Moreover, these mice accumulate a lipoprotein with many of the characteristics of β-VLDL, including a delayed in vivo turnover, and are therefore a bona fide model of human type III HLP.15

In the present study we used apo E(Cys142) transgenic mice to determine their susceptibility to atherosclerosis both on a normal-chow and on a high-fat, high-cholesterol diet. Transgenic mice developed only minimal lesions on a normal-chow diet, but after 3 months on a high-cholesterol diet they had lesion areas 10 times larger than their nontransgenic littermates. We conclude that the β-VLDL of type III HLP constitutes an atherogenic lipoprotein class and may be responsible for the development of atherosclerosis in human type III HLP subjects.

Methods

Nitrocellulose paper was purchased from Bio-Rad. The Superose 6 column, purchased from Pharmacia, was used on a Gilson Fast Performance Liquid Chromatography system. The Centricon filters were from Amicon. Cholesterol and triglyceride standards and reagents were from Abbott and Boehringer-Mannheim, respectively; the free cholesterol kit, Free Cholesterol C, was from Wakefield. Lipid determinations were performed on a Vmax microplate reader from Molecular Devices. Reagents for lipoprotein agarose gel electrophoresis were from Ciba Corning, the chemiluminescence detection kit for western blots was from Amersham, and Metofane (methoxyflurane) was from Pitman-Moore. The synthetic diet containing 1% cholesterol, 16% fat, and 0.5% cholic acid was custom-made by ICN.

Mice

Production of the line of apo E(Cys142) mice has been described.15 For the transgenic group we used only mice of the high-expressing line (ie, mice with approximately 70 mg/dL transgenic apo E in their plasma).15 These mice are hybrids of two inbred strains, C57BL/6J and SJL, and the lines were propagated by crossing F1 transgenic littermates with F1 nontransgenic littermates. The mice were kept in a 12-hour dark/light cycle (light from 7:00 AM to 7:00 PM), with males in individual cages and females in groups of 4 individuals per cage. In preparation for the diet study, mice had unrestricted access to food. At the end of the diet study blood was collected by cardiac puncture before perfusion of the heart and aorta for fixation. AM from nonfasted mice (access to food was unrestricted). At the University of California Committee on Animal Research. Phenylmethylsulfonylfluoride, a protease inhibitor. Plasma was obtained by centrifugation at 14,000 rpm (miniprep) for 15 minutes at 4°C, and samples were stored for no more than 2 days at 4°C in the presence of 1 mmol/L phenylmethylsulfonylfluoride, a protease inhibitor. Plasma samples of 100 μL were chromatographed on a Superose 6 column as previously described.16 For agarose gel electrophoresis, the major lipoprotein classes eluted from the column were pooled and concentrated using Centricon filters (fraction 15 through 19, VLDL; 20 through 22, IDL; 23 through 27, LDL and subclass 1 of high-density lipoproteins (HDL1); 28 through 32, HDL2).

After concentration, 5-μL aliquots were run on precast 1% agarose gels for 35 minutes at 90 V. The gels were either dried and stained with fat red 7B or transferred to nitrocellulose for a western blot assay using, sequentially, anti-rat and anti-human apo E antibodies. The human-specific antibody, produced in rabbit, showed very little cross-reactivity with mouse apo E (20 times lower than for human apo E). The rat-specific antisera, produced in rabbit, recognized mouse apo E and did not cross-react with human apo E. Ultracentrifugation was performed on plasma from individual mice (200 μL diluted to 500 μL in d<sub>1.006</sub> g/mL KBr solution), using a TL-100 Beckman table-top centrifuge and a TL-100.2 rotor (2.5 hours, 100,000 rpm, 10°C). The top 150 μL, representing the d<sub>1.006</sub> g/mL lipoprotein fraction, was used for agarose gel electrophoresis as described above and for protein electrophoresis on a 5% to 20% SDS-polyacrylamide gel and Coomassie staining or western blotting using mouse- and human-specific apo E antibodies.

Cholesterol and triglycerides were measured on total plasma and on ultracentrifuged and chromatographic fractions using the enzymatic colorimetric method adapted for use with a microplate reader.14 Cholesterol ester content of VLDL and β-VLDL was determined by difference after measurement of total cholesterol using the Boehringer-Mannheim kit, and that of free cholesterol was determined using the Free Cholesterol C kit.

Quantitation of Aortic Atherosclerosis

Aortic atherosclerosis was quantitated according to the method of Paigen et al. After euthanasia, blood was collected and the left ventricle of the heart cannulated and flushed, first with saline and then with 10% formalin in 0.075 mol/L phosphate buffer, pH 7.0. The inferior vena cava was cut to allow the perfusate to exit. The heart with attached aorta was removed, and frozen sections (10 μm thick) were taken, starting from the aortic valve, for a total length of 1200 μm. Sections were stained with oil red O for neutral lipids and hematoxylin to visualize the nuclei. The degree of atherosclerosis was evaluated on cryostat sections cut from a 340-μm length of aorta, beginning at the distal end of the aortic sinus. Every other section was analyzed, giving a total of 17 sections at intervals of 20 μm. Light microscopy images were captured digitally in full color by a Sony CCD-IRIS color video camera. Lesions were stained with oil red O and differentiated from background by color, and their areas were measured by automated pixel counting. Computer-aided image analysis was performed using an Image 1/AT system (Universal Imaging Corp). Results are reported as mean lesion area (in μm<sup>2</sup>) per section per animal.

Results

To investigate the spontaneous occurrence of atherosclerosis, 3 nontransgenic and 3 transgenic 4-month-old male mice were examined for the presence of atherosclerotic lesions in the proximal aorta. The mice had been fed regular mouse chow. As expected, no lesions were detectable in the aortic sections from nontransgenic mice. Transgenic mice had minimal lipid-stained microscopic lesions, localized on the aortic valve stumps. The lesions appeared to be very early fatty streaks that contained few cellular elements (data not shown). Although this minimal atherosclerosis clearly distinguished transgenic from nontransgenic mice, we tried to accelerate the atherosclerosis by feeding the mice a high-fat, high-cholesterol diet.
As shown in the Table, the high-fat, high-cholesterol diet induced an increase in plasma cholesterol level and a decrease in plasma triglyceride level. In both transgenic and nontransgenic mice, plasma cholesterol level increased during the first month on the diet (increasing by 60% and 31%, respectively) and then stabilized to a 20% increase in both groups at 3 months. Plasma triglyceride levels fell during the diet study, finally reaching 22% and 28% of their initial values in transgenic and nontransgenic mice, respectively.

In transgenic mice the increase in plasma cholesterol level during high-cholesterol feeding was due to a large increase in VLDL cholesterol, without significant changes in the HDL fraction. Fig 1 shows the cholesterol distribution among plasma lipoproteins of an apo E(Cys142) male mouse whose plasma cholesterol increased throughout the diet period. It is apparent that the major changes in cholesterol levels are restricted to VLDL (fractions 16 through 19) and, to some extent, IDL/LDL (fractions 20 through 27). In nontransgenic mice the increase in plasma cholesterol level was partly due to increased VLDL and LDL cholesterol levels and partly to increased HDL cholesterol level; moreover, HDL remained the major lipoprotein class (Fig 2). The diet period induced similar compositional changes in the VLDL of both nontransgenic and transgenic mice. In nontransgenic mice consuming normal chow less than 10% of VLDL cholesterol was in the esterified form, whereas in transgenic mice VLDL cholesteryl esters were about 30% of the total. In both types of mice, the diet induced an increase in VLDL cholesteryl esters to 68% of total cholesterol. These compositional changes were associated with changes in migration on agarose gel electrophoresis, upon which VLDL from both nontransgenic and transgenic mice showed a distinct β mobility (Fig 3). The high-fat, high-cholesterol diet induced increases in both endogenous and transgenic apo E in plasma by approximately 50%, and most of the extra apo E was associated with VLDL and IDL fractions (data not shown). However, in 2 of the 8 transgenic mice, the high-fat, high-cholesterol diet resulted in a decrease of approximately 50% in apo E(Cys142), as determined by western blot assay (data not shown).

The development of atherosclerotic lesions in the proximal aorta was assessed in 8 transgenic and 8 nontransgenic mice at the end of the diet period. The lesions observed in the transgenic mice included large areas of the aortic wall (Fig 4A) and also occurred on the aortic valve stumps. Furthermore, some of the lesions extended for more than 100 μm, were rich in cellular components such as foam cells, contained fibrous caps, and significantly thickened the artery wall. Some of the sections showed lesion formation in the coronary arteries, although never to the point of significantly narrowing the lumen. In contrast, the lesions in nontransgenic mice were very few in number, small in size, and low in cellular content (Fig 4B); moreover, these lesions were localized on the aortic valve stumps, an area where atherosclerotic lesions vary widely in size and have no association with aortic wall lesions."
At higher magnification, a raised atherosclerotic lesion (Fig 5A) from a transgenic mouse can be seen to contain oil red O–positive foam cells and several layers of superficial cells representing the early stage of a fibrous cap. In contrast, a comparable field of arterial wall from the aorta of a nontransgenic mouse is devoid of oil red O–positively stained material and lacks intimal lesions (Fig 5B). Fig 6 summarizes the quantitative results of the atherosclerosis study. When all mice in each group were considered, there was a 10-fold difference in the mean lesion area between transgenic and nontransgenic mice (15 416±7668 and 1419±938 μm² per section per animal, respectively; P<.09, Student’s t test). Lesion area varied widely in mice from both groups and was not associated with sex or prediet or postdiet cholesterol levels. After exclusion of the 2 transgenic mice with reduced postdiet levels of apo E(Cys142) that developed no atherosclerosis, the mean lesion areas in transgenic and nontransgenic mice were 20 554±9415 and 1419±938 μm² per section per animal, respectively (P=.02). Finally, iliac arteries from a subgroup of 4 transgenic mice were prepared and stained with oil red O, but no lipid-stainable areas were detected (data not shown).

Discussion

The most important feature of type III HLP in humans is that it predisposes affected individuals to premature development of atherosclerosis.14,22 We reasoned that the accumulation of β-VLDL in the plasma is most likely to be responsible for the increased atherogenic risk in type III HLP subjects. Therefore, the aim of the present study was to determine whether apo E(Cys142)–transgenic mice, which accumulate β-VLDL in the plasma and are therefore a model of type III HLP,13 would develop spontaneous and/or diet-induced aortic atherosclerosis. On a regular-chow diet, apo E(Cys142) transgenic mice had small fatty streaks on the aortic valve stumps, whereas nontransgenic mice had no signs of atherosclerosis. This difference was magnified by a 3-month high-fat, high-cholesterol diet. The diet increased plasma cholesterol level and decreased plasma triglyceride level in both transgenic and nontransgenic mice. However, the absolute levels of VLDL cholesterol in transgenic mice were 10 times higher than those in nontransgenic mice, and the HDL remained the major plasma lipoprotein in nontransgenic mice. As a consequence of these differences, transgenic mice had an atherosclerotic involvement of the proximal aorta 10 times greater than that of nontransgenic mice. The lesions extended over several sections, were raised above the lamina elastica, contained foam cells and cell debris, and were defined by an early-stage fibrous cap. No lesions were found at the bifurcation of the abdominal aorta with the iliac arteries, although this is a common location of atherosclerosis in humans with type III HLP.1 Diet-induced atherosclerosis has been recently demonstrated in transgenic mice coexpressing the defective variant apo E-Leiden with apo CI.23

This study identifies β-VLDL as the atherogenic component in type III HLP, although other factors must play a role in the susceptibility to atherosclerosis in this disorder. In fact, apo E–deficient mice, which have been produced in two laboratories using gene targeting techniques,24,25 develop spontaneous hyperlipidemia and extensive aortic atherosclerosis even when fed a regular chow diet. After 5 weeks on a western-type diet (21% fat, 0.15% cholesterol), apo E–deficient mice develop massive atherosclerosis in terms not only of aortic area involved but also of complexity of the lesions (eg, disruption of the lamina elastica, presence of cholesterol crystals and foam cells, and involvement of the coronary ostium).26–27

Several differences between apo E(Cys142) mice and the apo E–deficient mice might explain the different findings in these two animal models and provide some insights for the pathogenesis of human type III HLP due to apo E deficiency28 compared with that due to apo E mutations.1,2 First, the hyperlipidemia of apo E–deficient mice is mainly due to an elevation of cholesterol in VLDL, IDL, and LDL, whereas the apo E(Cys142) mice show an increase in cholesterol and a larger increase in triglycerides restricted largely to the VLDL fraction. It is not known whether the presence of triglycerides influences the atherogenic character of cholesterol-rich lipoproteins. However, it is possible that the lipoproteins of apo E(Cys142) mice are less atherogenic than those of apo E–deficient mice because they are richer in triglycerides. In fact, one of the major effects of the high-cholesterol diet, besides increasing plasma cholesterol level, was to significantly reduce plasma triglycerides, rendering the accumulating lipoproteins more similar in triglyceride content to those of apo E–deficient mice. This relatively lower triglyceride content, induced by the high-fat, high-cholesterol diet, was related to a significant development of atherosclerosis. Second, apo E(Cys142) mice have approximately normal amounts of endogenous apo E, which might counteract the deleterious effect of apo E(Cys142) and increase the clearance of smaller atherogenic lipoproteins compared with clearance in apo E–deficient mice; moreover, apo E(Cys142) still retains some ability to bind to the LDL receptor (about 25% residual activity in vitro compared with normal apo E3).14 Finally, in our apo E(Cys142) mice only the liver produces the transgenic defective apo E, while other tissues and cell types,
including macrophages, secrete only endogenous apo E. This expression pattern may be important in view of the role of macrophages in the artery wall in atherogenesis. One of the changes observed when the blood monocyte matures into a macrophage is the activation of apo E synthesis and secretion.\textsuperscript{29,30} Macrophage apo E secretion levels are associated with cholesterol efflux in the presence of an extracellular acceptor such as HDL\textsubscript{3}.	extsuperscript{31} It is possible that the relative protection from atherosclerosis in apo E(Cys142) mice on a normal-chow diet is due to the fact that they possess normal apo E-producing macrophages, whereas in apo E-deficient mice the macrophages are also apo E deficient.

We observed a large variability in the extent of atherosclerosis in nontransgenic and transgenic mice. For example, 2 of the 8 transgenic mice did not develop lesions after high-fat, high-cholesterol feeding. These mice also had a reduction in plasma apo E(Cys142) levels, although they appeared to be in good health during the diet experiment and did not have signs of liver disease at autopsy. Even after exclusion of these 2 mice from the quantitation of atherosclerosis, the variability was still high. Furthermore, there was no correlation between the extent of atherosclerosis and prediet or postdiet cholesterol levels or diet-induced changes in cholesterol levels. Plasma apo E(Cys142) levels were very similar in all transgenic mice except those mentioned above, suggesting that high levels of defective apo E may be necessary to induce atherogenesis but do not determine the extent of lesion development. We believe that this variability is due partly to the short period of high-fat, high-cholesterol feeding and partly to the fact that these mice were not backcrossed into a...
homogeneous genetic background, such as the C57BL/6J (atherosclerosis-susceptible) strain; instead, each had a different combination of genes from the C57BL/6J and SJL (atherosclerosis-resistant) strains. Because atherogenesis is a complex process in which many genes are probably involved, it is likely that the observed variability is due to the fact that each animal in this study had a unique combination of genes from the two parental strains, with a different distribution of atherosclerosis-susceptible and atherosclerosis-resistant genes. Variability in atherosclerotic response to diet has been observed by others (for review, see Reference 32). More recently, in their study of apo E-deficient mice, Plumet et al. reported a 10-fold difference in the extent of atherosclerosis.

Despite the fact that the VLDL from both nontransgenic and transgenic mice on the high-fat, high-cholesterol diet had β mobility (Fig 2) and increased levels of cholesterol and cholesteryl esters compared with the prediet period, transgenic mice had a 10-fold higher susceptibility to atherosclerosis (Fig 6). It has long been known that cholesterol feeding induces formation of β-migrating VLDL in several animal species, including the rat. These β-VLDL can be taken up by macrophages, stimulate cholesteryl ester deposition, and induce the formation of foam cells. It is not surprising that the β-VLDL of nontransgenic (normal) cholesterol-fed mice are less atherogenic than the β-VLDL from cholesterol-fed apo E(Cys142) mice. First, the increase in VLDL level and the accumulation of β-VLDL observed in transgenic mice were on average 10 times higher than in nontransgenic mice. Second, in nontransgenic mice the HDL were still the major plasma lipoproteins after the mice had been on the diet. The combination of these two factors might have contributed to the relative protection from atherosclerosis in nontransgenic mice. The ratio of β-VLDL to HDL is critical in modulating the accumulation of cholesteryl esters in macrophages, and a high β-VLDL:HDL ratio has been suggested as a major determinant of the susceptibility of cholesterol-fed dogs to the development of atherosclerosis. Furthermore, the presence of high amounts of apo E(Cys142) on the β-VLDL of transgenic mice might have increased the uptake of these lipoproteins by arterial wall macrophages, because apo E is the ligand for the β-VLDL recognition by the macrophage, a process that leads to formation of foam cells.

Overall, the results of this study implicate β-VLDL as an atherogenic lipoprotein in type III HLP, and further suggest that the apo E(Cys142)–transgenic mouse is a valid model of this disease. Furthermore, the apo E(Cys142)–transgenic mouse will be a useful model for pharmacological studies of control of the hyperlipidemia or the susceptibility to atherosclerosis in patients with type III HLP.

Acknowledgments

This work was supported in part by program project grant HL-47660 from the National Institutes of Health. The authors acknowledge Dale Newland for preparation and staining of the aortic sections; Howard Fein for measuring the lesion areas; Dr Karl H. Weisgraber for the gift of anti-human and anti-rat apo E antibodies and for a critical reading of the manuscript; Dawn Levy, Lewis DeSimone, and Sylvia Rich-mond for editorial work; and Liliana Jack and Amy Corder for preparation of the figures.

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Susceptibility to diet-induced atherosclerosis in transgenic mice expressing a dysfunctional human apolipoprotein E(Arg 112,Cys142).
S Fazio, D A Sanan, Y L Lee, Z S Ji, R W Mahley and S C Rall, Jr

*Arterioscler Thromb Vasc Biol.* 1994;14:1873-1879
doi: 10.1161/01.ATV.14.11.1873

*Arteriosclerosis, Thrombosis, and Vascular Biology* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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